Chapter 7

Extraction of DNA from Human Skeletal Material

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Abstract

In recent years the recovery and analysis of DNA from skeletal remains has been applied to several contexts ranging from disaster victim identification to the identification of the victims of conflict. Here are described procedures for processing the bone and tooth samples including mechanical and chemical cleaning, cutting and powdering in the presence of liquid nitrogen, complete demineralization of bone and tooth powder, DNA extraction, DNA purification using magnetic beads, and the precautions and strategies implemented to avoid and detect contamination. It has proven highly successful in the analysis of bones and teeth from Second World War victims' skeletal remains that have been excavated from mass graves in Slovenia and is also suitable for genetic identification of relatively fresh human remains.

Key words Bone, Teeth, DNA extraction, Second World War victims, Identification, Contamination

1 Introduction

In cases where unidentified skeletonized human remains are found and identification cannot be performed using classical forensic methods, bones or teeth can be used for molecular genetic identification. In bones and teeth binding of DNA to hydroxyapatite aids its preservation [1]. However, DNA does degrade with time and the environmental conditions (temperature, humidity, pH, geochemical properties of the soil, and the presence of microorganisms) determine the level of molecular preservation [2–4]. The key factors for DNA preservation are ambient temperature and humidity in which the skeletal remains were located since the time of the organism's death until their exhumation and subsequent molecular genetic testing. Highly stable environments with little annual fluctuation in temperature or humidity are favorable for DNA preservation. The best examples of DNA preservation can be found in samples located in caves or permafrost, where low temperatures provide the best possible conditions for preservation. Warm, wet environments dramatically increase the degradation of DNA, resulting in extensive damage and fragmentation [5, 6].

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Another aspect that affects the quality and quantity of DNA in skeletal remains is the storage method used after their exhumation [7, 8]. The effectiveness of genetic typing is much higher with freshly exhumed skeletons rather than with skeletons that have been kept at room temperature for several years, particularly because of higher ambient temperatures and washing of the skeletal remains before storing, which can reduce the pH and salt content of samples. Freezing the skeletal remains is preferred in order to ensure the best preservation of the DNA [9]. According to Fulton [10] the most appropriate protocol for long-term storage of old specimens varies depending on how the specimens were collected. If a sample was frozen upon collection, it is ideal to maintain that temperature. If a sample was collected at room temperature, it should be stored in a cool, dry environment and may not benefit from being frozen, in particular if several freeze/thaw cycles are anticipated. In general, simply avoiding environmental conditions that are known to promote DNA damage is a key to sample preservation. A cool, dry, temperature-stable environment is ideal. Avoid heat, freeze/thaw cycles, and moisture [10].

The condition of the skeletal remains analyzed for forensic identifications is often not ideal for DNA recovery. In old bones and teeth, small amounts of degraded endogenous DNA, the presence of polymerase chain reaction (PCR) inhibitors, and the exceptional risk of contamination limit the success of DNA typing [11–13]. Hydrolytic and oxidative damage are likely to affect DNA over time. Oxidative damage results in modified bases, whereas hydrolytic damage results in deamination of bases and in depurination and depyrimidination. Both mechanisms reduce the number as well as the size of the fragments that can be amplified by PCR [14]. Extraction methods used for obtaining the DNA have to avoid overly aggressive treatments, such as high temperatures or use of strong detergents to reduce further degradation of the already damaged ancient DNA [15]. Failure to amplify DNA may also result from the presence of inhibitory low molecular weight compounds that co-extract with DNA and inhibit DNA polymerase in PCR. Contamination with modern DNA represents another major limitation to the molecular analysis of old bones because, as a result of its higher concentration and quality, contemporary DNA amplification is favored over that of the endogenous DNA in the sample [14].

Nuclear DNA is the preferred genome of amplification for forensic purposes as it is individually specific and provides biparental kinship information [16]. In the past, mitochondrial DNA testing was regularly employed in the identification of aged skeletal remains [17–19]. Recently, some researchers (among them is also our group) have reported the successful typing of nuclear STRs from old skeletal material [20–25]. We managed to obtain nuclear DNA for successful STR typing from skeletal remains excavated

from the Auersperg chapel archaeological site that were over 300 years old [26]. In addition, we successfully identified victims of massacres that took place during and after the Second World War in Slovenia [27, 28]. Positive identification of the victims of Second World War mass graves was possible where we could collect reference samples from living relatives. Skeletal remains were analyzed for the Konfin I mass grave, located in a karst cave, where 88 victims were killed, the karst cave Konfin II mass grave with 62 excavated skeletons, and four mass graves found in the Storžič forest (4 victims), Bodovlje gorge (25 victims), Mozelj (5 victims), and Mačkovec (16 victims) where massacre victims were excavated.

The extraction method should remove as many inhibitors as possible and should gain the maximum available DNA [29]. Decalcification with 0.5 M ethylene diamine tetra acetic acid—EDTA enables separation of bone cells from the bone mass [30]. When working with fresh bones and teeth, decalcification is not needed. This step is very important for old skeletal remains, because decalcification is crucial for gaining higher quantities of DNA [31]. Loreille and coworkers [32] succeeded in gaining a sufficient quantity of DNA when they used complete demineralization from old skeletal remain that gave no results without it. As shown by the latest studies, total demineralization is the best method of DNA extraction from old bone material [33, 34], since total demineralization significantly increases the proportion of full profiles, reflecting a correlation with better DNA quality. According to benefits of demineralization our protocol is based on total demineralization.

The method of DNA extraction was developed in our laboratory to acquire high-quality DNA from Second World War skeletal remains and from skeletal remains from archaeological sites. The same method is also used in our laboratory for molecular genetic identification of unknown decomposed bodies in routine forensic casework where only bones and teeth are suitable for DNA typing. We analyzed 111 bones and teeth from Second World War mass graves to evaluate this method [35] and additionally 54 Second World War skeletal remains samples and some 300 years old bones and teeth from archaeological sites for change extraction protocol from partial to total demineralization [26, 36]. We analyzed 111 bones and teeth from Second World War victims using a partial decalcification method and extracted up to 55 ng DNA/g from teeth, up to 100 ng DNA/g from femurs, and up to 30 ng DNA/g from tibias. The typing of autosomal and Y-STR loci was successful in 95 % of the bones and teeth (there were approximately 20 % of partial profiles) and mtDNA in 96 % (HVSI) to 98 % (HVSII) of the samples analyzed [35]. Extracting genomic DNA using the total demineralization method from 54 Second World War skeletal remains samples gave us almost complete autosomal STR profiles in 52 out of the 54 samples; very few allelic drop-outs were observed in comparison to STR typing of extracts obtained with

partial demineralization [35, 36]. We successfully identified more victims of massacres that took place after the Second World War in Slovenia [27, 28]. We also performed an efficiency studies of different commercially available quantification and amplification kits for autosomal STR typing of skeletal remains excavated from the Second World War mass graves and concluded that they are highly reliable for human quantification and STR typing of old bones and teeth with the DNA extraction method optimized in our laboratory [36–38]. Amplification efficiency can be improved by addition of BSA (final concentration 40 ng/μl), which has the ability to bind to enzyme inhibitors present in DNA extract, and by increasing the number of amplification cycles. We used BSA in some bone and tooth extracts to overcome the PCR inhibition and we used three additional amplification cycles only in low-template bone and teeth DNA samples for STR DNA typing with commercially available amplification kits [28, 38]. When testing the performance of amplification kits with the extended European Standard Set (ESS) of loci on a sample of 102 seventy years old bones and teeth, DNA typing was successful in almost all of the samples. The method of DNA extraction described here has proved to be highly efficient because we obtained up to 131 ng DNA/g of bone and for the most of Second World War samples complete genetic profiles of autosomal STRs [37].

When drying the bones and teeth at 50 °C in an oven or incubator, the protocol yields DNA extracts in 28 h; otherwise two more days are needed to complete the extraction procedure when bone and tooth samples are dried at room temperature. The method proved effective from relatively small amount of bone or tooth powder (0.5 g). Less than 0.5 g can be used in case of small sample. In that case the volume of EDTA solution should be adjusted proportionally. The purification procedure using a Biorobot EZ1 (Qiagen) device doesn't use any aggressive organic solvents like phenol or chloroform. It is automated and takes only 20 min to complete. It is based on technology of magnetic particles that are covered with silicon. Such magnetic particles are very efficient for binding DNA, especially in the presence of chaotropic salts (like guanidine thiocyanate (GuSCN) or guanidine hydrochloride (GuHCl)). These salts are highly efficient for nucleic acid cleaning [39]. Chaotropic salts lyse cells, denature proteins, inactivate nucleases, and accelerate the binding of DNA to the paramagnetic particles covered with silicon. The whole extraction process is done in a huge filter tip that is thrown away after the procedure is finished. The rest of the extraction reagents are safely placed in a container—cartridge for single use only. Therefore, no manual pipetting is needed. This is very important for prevention of contamination. High efficiency of magnetic particles in DNA extraction was confirmed in several studies [40–43]. The purification using magnetic particles can be adapted also

to other robotic machines or can be performed manually using manual kits with magnetic particles from different suppliers. We successfully purified DNA from old skeletal material using also an AutoMate Express Instrument—Applied Biosystems [44].

Any validated human quantification and multiplex STR kit may be used with this extraction method. For autosomal STR typing of skeletal remains excavated from Second World War mass graves in Slovenia especially new amplification kits with the extended ESS loci proved highly reliable [36, 37]. Depending on the reference samples, different multiplex kits (autosomal STRs and Y-chromosomal STRs) and also sequencing of mtDNA may be needed to provide enough genetic markers for sufficiently significant results from genetic kinship analyses.

2 Materials

2.1 Chemicals

1. Sodium hypochlorite—bleach (Kemika); 6 % solution.
2. Alconox detergent (Sigma-Aldrich); 5 % solution.
3. Bi-distilled water (Sartorius-Stedim Biotech or Millipore).
4. Liquid nitrogen.
5. Ethanol (Merck); 80 %.
6. Ethylene diamine tetra acetic acid—EDTA (Promega); 0.5 M solution pH 8.0.
7. EZ 1 DNA Investigator Kit (Qiagen) contains:
   (a) Buffer G2
   (b) Proteinase K
8. DTT (Sigma-Aldrich); 1 M solution.
9. cRNA (EZ 1 DNA Investigator Kit, Qiagen).
10. Ultrapure distilled water HPLC grade (Gibco).
11. DNA Away™ (Molecular BioProducts).
12. HCl (Merck); 1 M solution.
13. Sodium acetate (Merck); 2 M solution pH 5.2.
14. NaOH (Merck); 5 M solution.

2.2 Consumable Goods

1. Filter tips.
2. pH indicator strips.
3. 50-ml Falcon tubes (Sarstedt).
4. 1.5- or 2-ml tubes (Eppendorf).
5. Scalpel blade.
6. Rough part of a dish sponge.
7. Plastic vial.

8. Cellulose (thin layer).
9. Sterile latex gloves.
10. EZ 1 DNA Investigator Kit (Qiagen) contains:
    (a) Cartridges with purification reagents
    (b) Sample tubes
    (c) Elution tubes
    (d) Filter tips with tip holders

2.3 Equipment

- Saw (Aesculap).
- Sterile saw blades (Aesculap).
- Standard laboratory equipment such as freezer and refrigerator for storing extracts and chemicals.
- Microbiological safety cabinet MC 3 (Iskra Pio).
- Laminar flow hoods MC 1 (Iskra Pio).
- Holding vice (Proxxon).
- High-speed grinding machine (Dremel).
- Circular diamond saws (Proxxon).
- Drilling bits (Proxxon).
- Shaker.
- Balance (A&D Company).
- Metal grinding vials (25 ml) with metal balls (2r = 20 mm) (Tehtnica—Domel).
- Bead Beater MillMix 20 (Tehtnica—Domel).
- Tweezers.
- Forceps.
- Mortuary needle.
- Hammer.
- Spatulas.
- Pipettes.
- Vortexes.
- Thermomixer comfort (Eppendorf) or any other shaker with temperature setting, suitable for use with 50-ml tubes.
- Centrifuge Megafuge 1.0 (Heraeus) or any other centrifuge suitable for use with 50-ml tubes.
- Instrument BioRobot EZ 1 (Qiagen).
- EZ 1 DNA Investigator Card (Qiagen).

3 Methods

3.1 Measures for Preventing and Detecting DNA Contamination

In the process of DNA typing, we encounter not only highly degraded DNA but also very small amounts of endogenous DNA which are very susceptible to contamination with modern DNA and are difficult to differentiate from the far more common modern (exogenous) DNA. Unfortunately, contamination is a serious

problem in investigations of DNA obtained from old skeletal remains [45–49]. Many chemical and physical environmental factors can have influence on contamination of skeletal remains with DNA from bacteria and fungi. That kind of contamination is not possible to prevent; however, it can affect the success of mitochondrial DNA and nuclear DNA typing. We are able to implement procedures that minimize the possibility of contamination with modern human DNA. Contamination of the endogenous DNA of bones and teeth with modern DNA can occur during exhumation, improper storage of the skeletal remains, and anthropological investigations [50]. Surface contamination can often occur due to improper handling of skeletal remains with bare hands. Contamination can also occur in the molecular genetic laboratory during the process of DNA typing where contaminating DNA can be located on the laboratory plastics and reagents. DNA fragments can also be present in the air in aerosol particles [51]. Therefore, it is necessary to consider the recommendations to prevent contamination. An elimination database, containing the profiles of the individuals that participated in the exhumation and subsequent analysis of the remains, can be used to check for authenticity of genetic profiles obtained from old skeletal remains and allows traceability in the case of contamination. Extraction negative controls have to be included in every extraction batch and PCR-negative controls in every amplification reaction to verify the purity of the extraction and amplification reagents and plastics. Doing so allows us to trace contamination in the event of its occurrence [52]. According to Rohland and Hofreiter [15] when processing more than seven bone or tooth samples, two or more extraction controls should be included in analyses. At least two samples must be typed for each skeleton, and it is necessary to obtain identical genetic profiles from both of them [52]. Physical separation of pre- and post-PCR areas is important to minimize the possibility of contamination through previously amplified products.

We eliminate surface contamination through different methods. The most important are washing in bi-distilled water, detergent, and ethanol; radiation with UV light and removing the bone surface and acquiring the bone or tooth material directly from the inside of the specimen. For successful decontamination, we usually use a combination of all listed methods. At the Laboratory of Molecular Genetics in the Institute of Forensic Medicine in Ljubljana, we follow stringent recommendations for prevention of contamination [1, 2, 52–62]. We use the following measurements to prevent contamination in the laboratory:

1. To prevent contamination with our own biological material, always use clean, sterile gloves (use double laboratory gloves) and change for every new sample. Use disposable surgical masks, caps, shoe covers, and disposable laboratory coats.

2. Clean the entire working surface before and after any work is performed with bleach (6 % sodium hypochlorite) followed by sterile bi-distilled water and 80 % ethanol, and/or nightly UV irradiation. Surfaces are cleaned in the same manner between each set of skeletal remains.
3. Clean all tools for cleaning, abrasion, and grinding of bones and teeth after use with bleach (6 % sodium hypochlorite) or with DNA Away™—Molecular BioProducts, which is very efficient in decontamination. Tools have to be exposed to the bleach for 15–30 min. Wash away the detergent with several washes with sterile bi-distilled water and 80 % ethanol and leave tools to air dry. Finally, all tools are sterilized using incubation at 134 °C for 45 min in a Tecno-Gaz sterilizer, and UV irradiated with shortwave (254 nm) UV source at least overnight or up to 72 h and for 30 min directly before starting to work.
4. Put all the reagents, tools, and laboratory plastics after sterilization under UV light at least overnight or up to 72 h. We expose all the listed material to UV light for 30 min directly before starting to work.
5. To avoid cross-contamination among samples a different set of equipment is used for each sample (such as grinding vials, cutting saw blades, drilling bits, tweezers, forceps, mortuary needles, and spatulas). These are cleaned and then stored in a way that makes it accessible but minimizes the possibility of cross-contamination with dust from cutting/grinding.
6. Take clean tools for each bone or tooth specimen.
7. Analyze bone and teeth samples separately from reference samples (e.g., for the elimination database). We use physically separated room for processing bone and tooth samples. Analyses of skeletons should be also temporally separated from reference samples and elimination database samples.
8. The separation of pre- and post-PCR procedures must be provided to prevent contamination with previously amplified products (all the equipment and protective clothing from post-PCR room never entered the pre-PCR laboratory). Amplified products from the post-PCR room should never be introduced to pre-PCR laboratory.
9. It is necessary to separate the dust-producing working steps from the contamination-susceptible steps like buffer preparation and PCR setup. We have different rooms in pre-PCR laboratory to separate each step in the bone typing procedure. We have room for cleaning and grinding the bones and teeth. In that room we clean the bones mechanically in a closed microbiological safety cabinet MC 3 (Iskra Pio) to capture and remove the bone powder that is released into the air during drilling and cutting. It has strong airflow to the filters that col-

lect the dust at the bottom of the chamber. The second room is used for preparation of buffers and solutions. The extraction room is used for decalcification, extraction, and purification, and the PCR room is used for the setup of PCR reagent mix (first hood) and addition of DNA extracts to the PCR (second hood). In each room we have laminar flow hoods with short-wave (254 nm) UV source and hepa filters. The laboratory setup must prevent dust from contaminating the rest of the process in DNA typing of skeletal remains.

10. Pre-PCR laboratory undergoes regular decontamination (washing with bleach, water, and ethanol). After the work the laminar flow hoods are irradiated at least overnight and for 30 min directly before starting to work.
11. To detect any possible contamination with DNA or previously amplified PCR products of reagents or laboratory plastics, we always use at least one negative PCR control.
12. For monitoring the cleanliness of the isolation reagents and laboratory plastics, and cross-contamination during the procedure we always use isolation negative control.
13. All genetic profiles obtained from skeletal remains are compared to elimination database.
14. Always use filter tips to minimize the risk of cross-contamination owing to DNA aerosols. Tips are exposed to UV light before use. Always use separate pipette tip for each sample to avoid cross-contamination.
15. We use the room for cleaning, grinding, decalcification, and extraction of DNA from bones and teeth exclusively for this kind of biological material and not for any other sample that contains high-template DNA (saliva, blood samples).
16. We isolate DNA from bones and teeth at least twice (from a different skeletal element of the same individual when possible) to check the results of genotyping and for interpretation reproducible results are used.

3.2 Reagent Preparation

All solutions should be prepared using HPLC-grade ultrapure water (Gibco) that was beforehand UV illuminated at least overnight.

3.2.1 Preparation of 5 % Alconox

Weigh 25 g of detergent Alconox (Sigma-Aldrich) on the balance into the 50-ml Falcon tube. Put it into 500-ml bottle, and add 500 ml ultrapure distilled water (Gibco). Put the bottle into hot water to dissolve the detergent. Expose the bottle to UV irradiation at least overnight or up to 72 h. Store at room temperature. The solution is stable for several months.

3.2.2 Preparation of 80 % Ethanol

Pour off 400 ml of absolute ethanol (Merck) into 500-ml bottle and add 100 ml ultrapure distilled water (Gibco). Expose the

bottle to UV irradiation at least overnight or up to 72 h. Store at room temperature. The solution is stable for several months.

3.2.3 Preparation of 0.5 M Ethylene Diamine Tetra Acetic Acid (EDTA) (pH 8.0)

Weigh 46.53 g EDTA (Promega) on the balance into 50-ml Falcon tube. Put it into 250- or 500-ml bottle and fill to 200 ml with ultrapure distilled water (Gibco). Adjust pH to 8.0 with 5 M NaOH. The final volume is 250 ml. Fill with ultrapure distilled water (Gibco) to 250 ml. Autoclave. Expose to UV irradiation at least overnight or up to 72 h. Store at room temperature. The solution is stable for several months.

To prepare 5 M NaOH: weigh 20 g NaOH (Merck) into a bottle and fill to 100 ml with ultrapure distilled water (Gibco) and autoclave. Store at room temperature. The solution is stable for several months.

3.2.4 Preparation of 1 μg/μl cRNA

Add 310 μl of ultrapure distilled water (Gibco) to lyophilized cRNA (included in the EZ 1 DNA Investigator Kit, Qiagen) and vortex at maximal speed for 10 s. Make aliquots—pipette 10 μl of the dissolved cRNA into 200-μl tubes. Deep freeze at −20 °C. Stored in the freezer it will remain stable for 6 months. Just before use take the frozen cRNA from the freezer and add it to the sample; discard the rest.

3.2.5 Preparation of 1 M DTT

Weigh 154 mg DTT (Sigma-Aldrich) in a 2-ml Eppendorf tube; add 1 ml ultrapure distilled water (Gibco) and 5 μl 2 M sodium acetate pH 5.2. Vortex, aliquot, and freeze at −20 °C. Just before use take the frozen 1 M DTT from the freezer. Stored in the freezer it will remain stable for 6 months.

To prepare 2 M sodium acetate: weigh 8.203 g anhydrides CH_3COONa (Merck) into 50-ml Falcon tube and fill to 50 ml with ultrapure distilled water (Gibco). Calibrate to pH 5.2 with 1 M HCl. Autoclave and store at room temperature. The solution is stable for several months.

To prepare 1 M HCl: put 10 ml concentrated HCl (Merck) into an autoclaved bottle and fill to 100 ml with ultrapure distilled water (Gibco). Don't autoclave. Store at room temperature. The solution is stable for several months.

3.3 Bone and Tooth Sample Preparation

3.3.1 Bone and Tooth Sample Selection

Long bones and teeth are the most appropriate samples for molecular genetic testing, as the DNA in them can stay well preserved for a long time [63–65]. The skull bones are the least suitable for genetic investigation according to Edson et al. [65]. From the comparative study of the performance of nuclear DNA typing of skeletal remains (we typed teeth, femurs, and tibiae but we didn't type any smaller elements of the hands and feet) from the mass graves of the Second World War, our laboratory discovered that teeth are the most suitable for typing, followed by the femur bones and tibiae [36, 37]. Similar conclusions were also reached by Miloš

et al. [63] and Misner and colleagues [64]. According to the experience of Keyser-Tracqui and Ludes [14], heavy (dense) bone is better than more brittle bone, which has lost lipid and collagen and has therefore increased porosity. Long bones (femur, tibia, and humerus) are preferred over rib or other thin bones and compact (cortical) bone is preferred to spongy bone. Rohland and Hofreiter [15] recommend getting the bone powder from diaphysis of long bones and use of dentine rather than enamel from teeth, as dentine is assumed to contain more DNA. Recently Mundorff et al. [66] and Mundorff and Davoren [67] found that smaller elements of the hands and feet (metatarsals, metacarpals, phalanges) were very similar or even better in DNA yield as both femora and tibiae. These bones can be easily sampled with a disposable scalpel, and thus reduce potential DNA contamination. Based on recent studies the current recommendations for preferential testing of long bones from the legs may need to be reevaluated and the sampling strategy for laboratories typing bone samples may change in the future.

The amount of DNA from individual and between groups of teeth varies strongly; quality and quantity of isolated DNA also depend on the tooth pathology, previous dental procedures, elapsed time since the extraction of the tooth until the isolation of DNA, and the donor's age [68]. Maximum DNA is obtained from the whole pulverized tooth, making it possible to capture DNA located in the hard dental tissues [69]. The amount of DNA depends on the size of the dental pulp and type of teeth; the molars are the richest source of DNA. Teeth suitable for DNA isolation appear in the following order: endodontically untreated molar, premolar, canine, and incisor and endodontically treated molar, premolar, canine, and incisor. At our laboratory, we select for genetic testing one long bone (preferably femur) and two teeth (preferably well-preserved and endodontically untreated molars) from each individual skeleton found in the Second World War graves; that is only possible through the excavation of skeletons in anatomic position. If excavation of skeletons was not carried out in the anatomical position, we select for molecular genetic investigations all left or all right femurs found in the grave. All skeletal material is photo-documented, appropriately labeled, and fragments of bones and teeth sampled for molecular genetic analyses are frozen at –20 °C until the DNA isolation procedure.

3.3.2 Bone and Tooth Sample Cleaning

Research has shown that washing and improper handling of remains contaminate their surface and can even penetrate into deeper layers. This kind of inner contamination is dependent on the stage of porosity and preservation of the remains [70–72]. Therefore, skeletal remains must be cleaned mechanically and chemically and teeth must be UV irradiated instead of mechanical cleaning. Although no procedures are 100 % efficient when removing contamination introduced during excavation, storage or

collection exists, cleaning improves the ratio between endogenous and contaminating DNA and it may reduce the amount of inhibitors introduced into the extraction [15].

We clean skeletal remains in a closed microbiological safety cabinet MC 3 (Iskra Pio) in a room designed exclusively for processing old skeletal remains. Bone samples are cleaned mechanically (physical removal of the surface using a rotary sanding tool (Dremel)) and chemically (washing in detergent, water, and ethanol), while tooth samples are cleaned chemically (washing in detergent, water, and ethanol) and irradiated with UV light for 2×30 min with the tooth rotated 180° between each exposure prior to grinding into a powder. To prevent bone warming during drilling and cutting, we frequently use liquid nitrogen to cool the bone and we use lower speed setting for abrasion and cutting. Warming of bone may cause degradation of endogenous DNA [59].

Between each sample all tools for drilling, cutting, and grinding of bones are cleaned by washing with water, bleach (6 % sodium hypochlorite) or DNA Away™ (Molecular BioProducts), sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore), and 80 % ethanol. Finally, everything is sterilized and UV irradiated at least overnight or up to 72 h. The steps for cleaning the bone and tooth samples are as follows:

1. With the use of a sterile clean saw blade cut 8–10 cm long and 2–3 cm wide fragment of femur just below trochanter (the same size can be applied for the rest long bones, too). Remove the molar with forceps from upper or lower jawbone. Store the bone fragment or tooth sample in the labeled 50-ml Falcon tube. If you don't work on that piece of bone or tooth sample immediately, freeze it at –20 °C for a long-term storage (*see* **Note 1**). The saw blade and the forceps must be changed for every new bone or tooth.
2. Remove dirt, soil, and any other material from the surface of the bone or tooth with a sterile scalpel blade, strong spatula, or needle (e.g., Mortuary needle) (*see* **Note 2**).
3. Put the bone or tooth into a plastic vial and with the use of a rough part of a sterilized and UV irradiated dish sponge wash it in sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore) with added mild detergent (add few ml of 5 % Alconox detergent to the water). Put the bone or tooth into 50-ml Falcon tube and wash it three times with sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore).
4. Dry the bone fragment or the tooth overnight; cover it with thin layer of cellulose paper (*see* **Note 3**).
5. Put everything you need for bone or teeth processing next day under UV light.

6. The next day weight bone or tooth sample and remove surface contamination with polishing (for teeth we use only UV irradiation on both side for 30 min). For removing surface contamination from the bone sample, use a closed microbiological safety cabinet. Fasten the dried bone into holding vice. With the high-speed grinding machine and drilling bit sand down surface 1–3 mm layer of the bone. Remove the surface layer from inner side of the bone fragment. Use liquid nitrogen to cool down the bone fragment and repeat the drilling on outer side of the bone.
7. Put mechanically cleaned bone into liquid nitrogen to cool.
8. Fasten the bone into holding vice. With the use of a circular diamond saw make notches in the shape of net (make small squares in dimension of 5×5 mm) on the outer surface of the bone. Use liquid nitrogen to cool down the bone fragment and make the same net also on the inner side of the bone (both sides have to look like a net).
9. Cut cleaned part of the bone with the circular diamond saw from the rest of the bone and put it into a sterile 50-ml Falcon tube using sterile tweezers. **Steps 6–9** are performed in a fume hood to control dust. The weight of cleaned piece of bone is approximately 2–3 g. The rest of the bone sample store for additional extraction if needed. The next step is chemical cleaning of bones and teeth with 5 % Alconox detergent (Sigma-Aldrich), sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore), and 80 % ethanol.
10. Wash out the bone or tooth sample for 1 min with bi-distilled water (Sartorius-Stedim Biotech or Millipore). Mildly shake it.
11. Pour off water from the Falcon tube.
12. Wash the bone or tooth sample with 5 % Alconox detergent (Sigma-Aldrich) and mildly shake it for 15–30 s on a shaker. Pour off the detergent from the Falcon tube (*see* **Note 4**). To remove detergent four steps of washing follow.
13. Wash the bone or tooth sample with bi-distilled water (Sartorius-Stedim Biotech or Millipore) for 3 min and mildly shake it.
14. Pour off water from the Falcon tube.
15. Repeat washing with water once again.
16. Wash the bone or tooth sample in 80 % ethanol for 30 s and mildly shake it.
17. Pour off ethanol from the Falcon tube.
18. Repeat washing with ethanol once again.
19. Dry clean fragment of the bone or tooth sample overnight and cover it with thin layer of cellulose paper (*see* **Note 3**).
20. All listed steps are done to remove possible contaminants from previous handling from the sample surface.

21. Clean the entire working surface of a closed microbiological safety cabinet after the work or between working with different skeletal remains with bleach (6 % sodium hypochlorite), sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore), and 80 % ethanol using paper towels.
22. Put everything you need for your work next day under UV light.
23. Next day put the dried bone or tooth sample into 50-ml Falcon tube and weigh it on the balance and write down the results on Falcon tube.

3.3.3 Bone and Tooth Sample Powdering

It is necessary to obtain very fine bone or tooth powder to extract enough quantity of DNA from old skeletal remains. Demineralization is better and faster with very small pieces of powder, so generate as fine powder as possible to maximize the surface area of the sample that will eventually contact the chelation solution [15]. We use homogenizer Bead Beater MillMix 20 (Tehtnica—Domel) and liquid nitrogen to obtain fine bone and tooth powder. We cool metal vials and bone or tooth samples in liquid nitrogen to avoid overheating during powdering and then we grind them for 1–2 min at 30 Hz. The powder we get is transferred into a sterile 50-ml Falcon tube. We weight the powder and use it in the extraction process. We pulverize skeletal remains in a room designed exclusively for processing old skeletal remains. Grinding vials need to be thoroughly cleaned before reuse. Between each sample, they have to be cleaned by washing with water, bleach (6 % sodium hypochlorite) or DNA Away™ (Molecular BioProducts), sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore), and 80 % ethanol. Finally, the grinding vials are sterilized and UV irradiated at least overnight or up to 72 h and additionally 30 min before use. A sufficient number of grinding vials are required to prepare more than one sample per day. The steps for powdering the bone and tooth samples are as follows:

1. Pour liquid nitrogen over the bone or tooth sample in Falcon tube and wait until it evaporates.
2. Pour liquid nitrogen into the lower part of the metal vial and after evaporation repeat twice to cool the grinding vial.
3. With help of tweezers move the bone or tooth sample into the sterile and UV irradiated glove finger, wrap it in several sterile cellulose papers, and break it into smaller pieces with hammer (bone will break on the notches previously made with circular diamond saw).
4. Move the broken pieces in dimension of 5×5 mm with tweezers into the cooled lower part of the metal vial. Add metal ball and close the vial with its own metal lid. Grind to obtain bone powder at 30 Hz 1–2 min in the Bead Beater MillMix 20 (Tehtnica—Domel).

5. Move the bone or tooth powder into the sterile 50-ml Falcon tube and weigh it on the balance. Write the result on the Falcon tube.
6. The sample powder can be stored at 4 °C while in use, but should be subjected to the extraction as soon as possible. For long-term storage store it at –20 °C.

3.3.4 Bone and Tooth Sample Decalcification

Genomic DNA is obtained from 0.5 g of bone or tooth powder incubated in 10 ml of 0.5 M ethylene diamine tetra acetic acid—EDTA pH 8 overnight at 37 °C for decalcification. EDTA is a strong chelator that is able to bind metallic ions such as calcium in the bone or tooth powder and allows for its removal. High amounts of EDTA are necessary to dissolve part of the hydroxyapatite matrix specific to bone and teeth samples [15]. For total demineralization 15 ml of 0.5 M EDTA per g of bone or tooth powder is needed. That amount of EDTA can theoretically bind only the amount of calcium contained in 1 g of bone or tooth powder [32]. At the end of decalcification process, the precipitate is washed with sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore). The steps for decalcification of bone or tooth samples are as follows:

1. Weigh 0.5 g of bone or tooth powder in the 50-ml Falcon tube. Use sterile spatula.
2. Prepare another 50-ml Falcon tube used for isolation blind control. Put reagents for DNA isolation in this tube only (do not put bone or tooth powder in this tube). Blind control has to be treated identically to the experimental samples throughout the procedure.
3. Add 10 ml of 0.5 M EDTA and vortex for 10 s (*see* **Note 5**).
4. Incubate bone or tooth powder and extraction negative control overnight at 37 °C and mix at 750 rpm on the Thermomixer comfort (Eppendorf).
5. Put the necessary material for working on the bone or tooth next day under UV light.
6. Centrifuge at 1300 × *g* for 15 min, a pellet of residual powder that is typically seen at this point.
7. Pipette and discard whole supernatant. In extraction negative control leave only approximately 100 µl of supernatant.
8. Add 10 ml of sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore) and vortex at high speed for 10 s. Add 10 ml of sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore) also to the extraction blind control sample.
9. Centrifuge at 1300 × *g* for 15 min.
10. Pipette and discard whole supernatant (for the blind control leave only approximately 100 µl of supernatant).

3.4 DNA Extraction and Purification

We don't use the organic extraction with phenol/chloroform/isoamyl alcohol for extraction and purification of DNA. In our method the DNA is purified in a Biorobot EZ1 (Qiagen) device using the EZ1 DNA Investigator Card and EZ1 DNA Investigator Kit (Qiagen) (*see* Chapter 5). Both phenol and chloroform are dangerous and treatment with them should always be performed in a vented fume hood. Because of their toxicity, it is much safer to use other efficient methods of purification of DNA. We optimized extraction and purification of DNA in Biorobot EZ1 device (Qiagen) to obtain sufficient amount of bone and tooth DNA for successful STR typing.

3.4.1 Extraction of DNA

An extraction buffer, proteinase K, and DTT are added to the precipitate and incubated for 2–3 h at 56 °C. Higher incubation temperature improves the digestion of the bone or tooth powder and thereby releases more DNA, especially in cases when the powder used is relatively coarse (*see* **Note 6**). Proteinase K is an endolytic serine protease that cleaves proteins, reducing them to their constituent amino acids. DTT is a reducing agent that can cleave cysteine-cystine bridges and disrupt the tertiary structure of some proteins and allow further degradation. The steps for extraction of DNA from bone or tooth samples are as follows:

1. Add 100 µl G2 buffer (EZ 1 DNA Investigator Kit, Qiagen) to the pellet and to the blind control.
2. Add 60 µl Proteinase K (EZ 1 DNA Investigator Kit, Qiagen) to the pellet and to the blind control (*see* **Note 7**).
3. Add 20 µl 1 M DTT to the pellet and to the blind control (*see* **Note 8**).
4. Vortex for 10 s (*see* **Note 5**).
5. Incubate at 56 °C and 750 rpm in the Thermomixer comfort (Eppendorf) for 2–3 h.

3.4.2 Purification of DNA

After centrifugation, the supernatant is taken to purify the DNA in a Biorobot EZ1 device (Qiagen). The DNA extract should be colorless. We store the DNA that was extracted until forthcoming steps of quantification and genotyping of nuclear and mtDNA at 4 °C while in use and at −20 °C for long-term storage. DNA is susceptible to damage from repeat freeze-thaw cycles and should be defrosted as infrequently as possible [73]. We always include negative controls in the process of extraction to check cleanliness of laboratory plastics and reagents. The steps for purification of DNA from bone or tooth samples are as follows:

1. Centrifuge the bone or tooth lysate and the blind control sample at $600 \times g$ for 2 min.
2. Pipette 200 µl (for the blind control sample) and 400 µl (for the bone or tooth lysate) of the supernatant into sample tube

(Qiagen). Keep the remaining supernatant (up to 300 μl); you may wish to retain it for a second round of extraction with Biorobot EZ1 device. Store the remaining lysate in a 1.5- or 2-ml Eppendorf tube and freeze it at –20 °C (*see* **Note 9**).

3. Add 1 μl cRNA—Qiagen (concentration of 1 μg/μl) (*see* **Note 8**).
4. Put the sample tubes into Biorobot EZ1 instrument (Qiagen).
5. Choose: trace protocol, water elution, and 50 μl volume of elution in the software menu of Investigator software Card (Qiagen).
6. After the automated purification procedure, you get 50 μl isolated DNA sample. Close elution tubes with extracted DNA and blind control sample and store them at 4 °C while in use and then place it at –20 °C for long-term storage (*see* **Note 10**).

4 Notes

1. According to Rohland and Hofreiter [15], the sample can be stored at room temperature, but we prefer to be cautious and store at –20 °C.
2. This step is important because dirt may introduce a variety of inhibitory substances to the extraction procedure, and therefore to the extract itself; these substances may interfere or even completely block subsequent enzymatic manipulations of the DNA extracts [15].
3. It is also possible to dry bones and teeth at 50 °C for 2 h in an oven or incubator.
4. Any detergent carryover will degrade the DNA and reagents in subsequent steps of the DNA extraction; thus it is extremely important that detergent is removed completely.
5. Vortex at slow speed to prevent sticking of pellet on the walls of Falcon tube.
6. Be aware that higher temperature may cause further damage or degradation of the DNA [15].
7. In our protocol we use more proteinase K than it is recommended by the manufacturer and supplied with EZ 1 DNA Investigator Kit (Qiagen). Accordingly some extra proteinase K (Qiagen) has to be ordered.
8. Thaw it immediately before use.
9. The second extraction will usually contain lower amount of DNA compared to the first and you can use the first one for nuclear DNA typing and the second one for mtDNA typing.
10. It may be useful to subdivide the final extract into aliquots of 10 μl and to use these as necessary (successive freeze-thaw cycles can damage the DNA over time).

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